REMARKS

Claims 1-13 and 25-36 are currently pending in the application. Claim 1 is amended herein to incorporate the limitations of claims 25 and 26. Claims 1 and 13 are amended to specify that microvessel outgrowth from said placental stem cells or said vessel section occurs. Support for these amendments is found in the specification at least in the Summary of the Invention and the Examples. Claims 27 and 28 are amended to depend from claim 1 rather than claim 25. Claim 36 is amended herein to depend from claim 35 rather than claim 34. No new matter is added by these amendments. Claims 25, 26, 29 and 30 are canceled herein without prejudice to Applicants' right to pursue the subject matter of these claims in a related application. Upon entry of the present Amendment, claims 1-13, 27, 28 and 31-36 will be pending.

The Rejections Under 35 U.S.C. § 112, Second Paragraph Should Be Withdrawn

The Examiner has rejected claims 1-13 and 25-36 under 35 U.S.C. § 112, second paragraph on several bases as indefinite. Office Action at pages 5-6. Applicants address each as follows.

"The test for definiteness under 35 U.S.C. 112, second paragraph, is whether 'those skilled in the art would understand what is claimed when the claim is read in light of the specification.'" Manual of Patent Examining Procedure, Eighth Edition Incorporating Revision No. 6 ("MPEP") § 2173.02, at 2100-219 (citation omitted). A claim is indefinite only when it "remains insolubly ambiguous without discernible meaning after all reasonable attempts at construction . . ." *Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings.*, 370 F.3d 1354, 1366 (Fed. Cir. 2004); *cert. denied*, 126 S. Ct. 2976 (2006). A claim is definite if it provides clear warning to others as to what constitutes infringement of the patent. *See, e.g., Solomon v. Kimberly-Clark Corp.*, 216 F.3d 1372, 1379 (Fed. Cir. 2000).

The Examiner has rejected claims 1-12, 31 and 33-38 as indefinite in the recitation of "a plurality of stem cells." A person of ordinary skill in the art would understand the term "plurality" to mean two or more stem cells. This construction is common. For example, a search for the phrase "a plurality" in the claims of published applications on the United States Patent and Trademark Pregrant Publication Database Search webpage returns over 2100 published applications, as of March 5, 2008, which use the phrase "a plurality." As such, the claims are not indefinite on this basis. The Examiner further contends that "the claim does not define the phenotype of the stem cell of the origin of the stem cell . . ." Office Action at page 5. The Examiner further states that "the claim does not define the phenotype of the stem

cell or the origin of the stem cell . . . [c]ould any stem cell of any phenotype be induced to differentiate into a microvessel under culture conditions?" Without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to specify that the recited stem cells are $CD34^-$ placental stem cells, mesenchymal stem cells or bone marrow-derived stem cells. The claims as amended are definite.

The Examiner also contends that claims 1-13 and 25-33 are indefinite in the recitation of "said control level" in claims 1 and 13. Office Action at page 5. Without conceding the propriety of the Examiner's rejection, Applicants have amended claims 1 and 13 to recite "control amount" rather than "control level."

The Examiner further contends that claims 1 and 13 omit a step identifying the correlation or relationship between endothelial cell growth and microvessel outgrowth. Office action at pages 5-6. Without conceding the propriety of the Examiner's rejection, Applicants have amended claims 1 and 13 to specify microvessel outgrowth from the recited stem cells (claim 1) or vessel section (claim 13) occurs. Applicants have also amended claims 1 and 13 to no longer recite "for a time."

The Examiner further contends that claim 3 is indefinite for the recitation of "a plurality of tumor cells." Office Action at page 6. As explained above, "plurality" indicates "two or more." Thus, a person of skill in the art would readily appreciate the scope of claim 3 so as to be able to avoid infringement.

Finally, the Examiner contends that claim 36 is indefinite in the recitation of "said stimulator of angiogenesis." Applicants have amended claim 36 to depend from claim 35, which provides appropriate antecedent basis for this limitation.

For the above reasons, Applicants respectfully request that the Examiner withdraw these rejections of the claims.

The Rejections Under 35 U.S.C. § 102(e) Should Be Withdrawn

The Examiner has rejected claims 1-9, 12, 13, 29, 30 and 34-36 under 35 U.S.C. § 102(e) as allegedly anticipated by Drake *et al.*, WO 01/63281 ("Drake"). Office Action at pages 7-9. To establish anticipation under 35 U.S.C. § 102(b), the Examiner must establish that each and every limitation of the claim is disclosed in the cited reference, either expressly or inherently. *See Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Claims 29 and 30 have been canceled herein without prejudice.

Without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to incorporate the limitations of claims 25 and 26, which were not rejected

by the Examiner on this basis. As amended, claim 1, and claims 2-9. 12. 29, 20 and 34-36, all of which ultimately depend from amended claim 1, are not anticipated by Drake, because Drake does not disclose a method of identifying a modulator of angiogenesis or vasogenesis comprising culturing a plurality of isolated human $CD34^-$ placental stem cells in the presence of a test compound.

With respect to claim 13, Drake fails to teach an assay to identify a modulator of angiogenesis, wherein a vessel section is cultured in the presence of a plurality of tumor cells and a test compound. The Examiner contends that "Drake teaches assays using human breast carcinoma cell lines (Example 10)" (Office Action at page 9). However, Example 10 is a prophetic example directed only to an assay to determine the effect of tumor cells on angiogenesis and vasculogenesis in an irradiated mouse. Example 10 does not *demonstrate* that tumor cells have any effect on vasculogenesis or angiogenesis, and does not demonstrate a method of identifying modulators of angiogenesis. Moreover, although the Examiner cites page 12, lines 28-29 as demonstrating that "the method substrate can include . . . tumor," this statement in Drake is in the context only of the definition of the term "contacting". As is clear from other passages in the specification, "contacting," in the context of tumor cells, is only contacting of agents that have *already* been identified as angiogenesis modulators with tumor cells. *See, e.g.*, Drake at page 4, lines 10-19; page 14, line 30 to page 15, line 7; and page 16, lines 10-13. As such, Drake does not disclose the use of tumor cells in the assays as presently claimed.

Applicants respectfully request that the Examiner withdraw this rejection of the claims.

The Rejections Under 35 U.S.C. § 103 Should Be Withdrawn

The Examiner has rejected claims under 35 U.S.C. § 103 as obvious over several different combinations of references. Applicants traverse each of the rejections separately below.

A determination of obviousness requires analysis of "1) 'the scope and content of the prior art'; 2) the 'differences between the prior art and the claims'; 3) 'the level of ordinary skill in the pertinent art'; and 4) 'objective evidence of nonobviousness'." *KSR International Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1734 (2007) (quoting *Graham v. John Deere & Co. of Kansas City*, 86 S. Ct. 684 (1966)). In rejecting a claim for obviousness, an Examiner "must explain why the difference(s) between the prior art and the claimed invention would have been obvious to one of ordinary skill in the art." "Examination Guidelines for Determining

Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in *KSR International Co.* v. *Teleflex Inc.*," 72 Fed. Reg. 57526, 57528 (2007) ("Guidelines"); *see also* MPEP, Section 2100 at page 2100-118. Applicants note that the "teaching, suggestion or motivation" element was not obviated by *KSR*; *see, e.g., Takeda Chemical Indus., Ltd. v. AlphaPharm Pty., Ltd.*, 492 F.3d 1350, 1356 (Fed. Cir. 2007). Additionally, "[r]ejections on obviousness cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." Guidelines at pages 57258-59, citing *KSR*; *see also* MPEP at page 2100-119, left column. In reviewing the claims for obviousness, the Examiner must avoid impermissible hindsight, and determine the obviousness of the invention to a person of skill in the art at the time the invention was made, based only on the prior art and not on Applicant's disclosure. *See* MPEP at page 2100-121 and 2100-127.

The Rejection Over Drake and Fox

The Examiner has rejected claims 1 and 36 as obvious over Drake view of Fox *et al.*, *J. Pathol.* 179:232-237 (1996) ("Fox"). Office Action, pages 10-12. As noted above, without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to incorporate the limitations of claims 25 and 26, which were not rejected by the Examiner on this basis. Moreover, claim 36 has been amended to depend from claim 35. As explained above, Drake does not teach or suggest a method of identifying a modulator of angiogenesis or vasogenesis comprising culturing a plurality of human placental stem cells in the presence of a test compound. Fox fails to remedy this deficiency, because Fox, as cited, does not disclose either a method of identifying modulators of angiogenesis or the use of placental stem cells. Therefore, a person of ordinary skill in the art could not, without undue experimentation, combine the disclosures of Drake and Fox to produce the claimed method. As such, claims 1 and 36, as amended, are not obvious in view of Drake and Fox.

The Rejection Over Drake and Montesano

The Examiner has rejected claims 1, 10 and 11 as obvious over Drake view of Montasano *et al.*, *J. Cell. Physiol.* 132(3):509-516 (1987) ("Montasano"). Office Action at pages 12-13. As noted above, without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to incorporate the limitations of claims 25 and 26, which were not rejected by the Examiner on this basis. As explained above, Drake does not teach or suggest a method of identifying a modulator of angiogenesis or vasogenesis comprising culturing a plurality of human placental stem cells in the presence of a test compound.

Montasano fails to remedy this deficiency, because Montasano, as cited, does not disclose either a method of identifying modulators of angiogenesis or the use of placental stem cells. Therefore, a person of ordinary skill in the art could not, without undue experimentation, combine the disclosures of Drake and Montasano to produce the claimed method. As such, claims 1, 10 and 11, as amended, are not obvious in view of Drake and Montasano.

The Rejection Over Drake and Zygmunt

The Examiner has rejected claims 1 and 25-28 as obvious over Drake in view of Zygmunt, *Early Pregnancy* 5(1):72-73 (2001). Office Action at pages 13-15. As noted above, without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to incorporate the limitations of claims 25 and 26. Claims 25 and 26 have been canceled herein.

The Examiner has not made out the required *prima facie* case of obviousness. First, the Examiner has not established that angioblasts or hemangioblasts—endothelial progenitor cells, the only types of cells mentioned by Zygmunt—are the placental stem cells recited in the claims. The Examiner contends that "Zygmunt discloses that placental vascularization . . . is mediated by endothelial progenitor cells present in the developing primitive organ." Office Action at page 14. Though conceding that "Zygmunt does not describe the phenotype of the *placental stem cells* for endothelial progenitors (emphasis added)," the Examiner contends that one of ordinary skill in the art would "envisage" that the "endogenous placental stem cells inherently" express the markers specified in claims 27 and 28. The Examiner cites no reference or authority to support what a person of ordinary skill in the art would "envisage," or to support a rejection based on inherency. The Examiner must provide a rationale or evidence tending to show inherency. MPEP, Section 2100 at page 2100-47. The Examiner must also show that the missing feature would *necessarily* be present in the thing described in the cited art, and that it would be so recognized by persons of ordinary skill in the art. *Id*, right column. The Examiner has not established either that the markers recited in the claims are necessarily present on the cells allegedly disclosed in Zygmunt or in Drake, or that a person of skill in the art would recognize them to be present. As such, the assertion that the cells of Zygmunt display the recited cellular marker characteristics is a conclusory statement that cannot support obviousness. *See* Guidelines at pages 57258-59. The Examiner has thus not established that the combination of Drake and Zygmunt teaches each of the limitations of the claimed methods, or that a person of ordinary skill in the art, combining the two references, could practice the claimed method without undue

experimentation. As such, the Examiner has not made out the requisite *prima facie* case of obviousness.

Moreover, the angioblasts and hemangioblasts mentioned in Zygmunt are not the placental stem cells recited in the claims. For example, it is known that angioblasts and hemangioblasts are $CD34^+$. *See, e.g.*, Urbich, "Endothelial Progenitor Cells," *Circulation Research* 95(4):343-53 (2004), a copy of which is attached hereto, at least at page 344, left column. However, as amended, claim 1, and claims dependent therefrom, recite the use of $CD34^-$ placental stem cells. Moreover, angioblasts are not *stem cells*, as required by the claims; angioblasts are only progenitor cells in that they only develop into endothelial cells. Clearly, the claimed method is directed to the use of different cells than disclosed by Zygmunt. The claimed invention therefore cannot be derived from the cited references. Instead, only through review of Applicants' specification would it become apparent that placental stem cells can be used in the claimed method. The Examiner, therefore, has necessarily used impermissible hindsight in framing this rejection.

Moreover, Zygmunt merely mentions that angioblasts exist in the developing placenta. Zygmunt fails to disclose how to isolate such cells into a form useful in the claimed assay. As such, because placental stem cells are not angioblasts or hemangioblasts, and because the cells disclosed in Zygmunt are not isolated, a person of ordinary skill in the art would have to perform an unreasonable amount of experimentation, and would have no reasonable expectation of success, in combining Drake and Zygmunt to practice the claimed method.

Therefore, the cited combination of references fails to teach a method of identification of a modulator of angiogenesis comprising contacting placental stem cells with a test compound, as recited in the amended claims.

The Rejection Over Drake and Crouse

The Examiner has rejected claims 1 and 5 as obvious over Drake view of Crouse *et al.*, *Kroc Found. Ser.* 18:211-231 . Office Action at pages 15-17. As noted above, without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to incorporate the limitations of claims 25 and 26, which were not rejected by the Examiner on this basis. As explained above, Drake does not teach or suggest a method of identifying a modulator of angiogenesis or vasogenesis comprising culturing a plurality of isolated human placental stem cells in the presence of a test compound. Crouse fails to remedy this deficiency, because Crouse, as cited, does not disclose either a method of identifying

modulators of angiogenesis or the use of placental stem cells. Therefore, a person of ordinary skill in the art could not, without undue experimentation, combine the disclosures of Drake and Crouse to produce the claimed method. As such, claims 1, 10 and 11, as amended, are not obvious in view of Drake and Crouse.

The Rejection Over Drake and Merrick

The Examiner has rejected claims 1, 2 and 33 as obvious over Drake view of Merrick *et al.*, *Transplantation* 62(8):1085-1089 (1996) ("Merrick"). Office Action at pages 17-18. As noted above, without conceding the propriety of the Examiner's rejection, Applicants have amended claim 1 to incorporate the limitations of claims 25 and 26, which were not rejected by the Examiner on this basis. As explained above, Drake does not teach or suggest a method of identifying a modulator of angiogenesis or vasogenesis comprising culturing a plurality of human placental stem cells in the presence of a test compound. Merrick fails to remedy this deficiency, because Merrick, as cited, does not disclose either a method of identifying modulators of angiogenesis or the use of placental stem cells. Therefore, a person of ordinary skill in the art could not, without undue experimentation, combine the disclosures of Drake and Merrick to produce the claimed method. As such, claims 1, 10 and 11, as amended, are not obvious in view of Drake and Merrick.

CONCLUSION

Applicant respectfully requests that the present remarks be made of record in the file history of the present application. An early allowance of the application is earnestly requested. The Examiner is invited to contact the undersigned with any questions concerning the application. No fee is believed to be due for this Preliminary Amendment. Should any fee be required, please charge the necessary amount to Jones Day Deposit Account No. 50-3013

Respectfully submitted,

Date: March 5, 2008

By: Lawrence S. Graham Reg. No. 49,020
For: Anthony M. Insogna Reg. No. 35,203

JONES DAY
222 East 41st Street
New York, New York 10017
(212) 326-3939

This Review is part of a thematic series on Angiogenesis, which includes the following articles:

Endothelial Progenitor Cells: Characterization and Role in Vascular Biology

Bone Marrow–Derived Cells for Enhancing Collateral Development: Mechanisms, Animal Data, and Initial Clinical Experiences

Arteriogenesis

Innate Immunity and Angiogenesis

Syndecans

Growth Factors and Blood Vessels: Differentiation and Maturation

Ralph Kelly, Guest Editor

Endothelial Progenitor Cells
Characterization and Role in Vascular Biology

Carmen Urbich, Stefanie Dimmeler

Abstract—Infusion of different hematopoietic stem cell populations and ex vivo expanded endothelial progenitor cells augments neovascularization of tissue after ischemia and contributes to reendothelialization after endothelial injury, thereby, providing a novel therapeutic option. However, controversy exists with respect to the identification and the origin of endothelial progenitor cells. Overall, there is consensus that endothelial progenitor cells can derive from the bone marrow and that CD133/VEGFR2 cells represent a population with endothelial progenitor capacity. However, increasing evidence suggests that there are additional bone marrow–derived cell populations (eg, myeloid cells, "side population" cells, and mesenchymal cells) and non-bone marrow–derived cells, which also can give rise to endothelial cells. The characterization of the different progenitor cell populations and their functional properties are discussed. Mobilization and endothelial progenitor cell–mediated neovascularization is critically regulated. Stimulatory (eg, statins and exercise) or inhibitory factors (risk factors for coronary artery disease) modulate progenitor cell levels and, thereby, affect the vascular repair capacity. Moreover, recruitment and incorporation of endothelial progenitor cells requires a coordinated sequence of multistep adhesive and signaling events including adhesion and migration (eg, by integrins), chemoattraction (eg, by SDF-1/CXCR4), and finally the differentiation to endothelial cells. This review summarizes the mechanisms regulating endothelial progenitor cell–mediated neovascularization and reendothelialization. (***Circ Res.*** **2004;95:343-353.)**

Key Words: progenitor cells ■ neovascularization ■ vasculogenesis ■ angiogenesis ■ endothelial cells

Differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation was believed to exclusively occur in embryonic development. This dogma was overturned in 1997, when Asahara and colleagues[1] published that purified $CD34^+$ hematopoietic progenitor cells from adults can differentiate ex vivo to an endothelial phenotype. These cells were named "endothelial progenitor cells" (EPCs), showed expression of various endothelial markers, and incorporated into neovessels at sites of ischemia. Rafii's group in 1998[2] also reported the existence of "circulating bone marrow–derived endothelial progenitor cells" (CEPCs) in the adult. Again, a subset of $CD34^+$ hematopoietic stem cells was shown to differentiate to the endothelial lineage and express endothelial marker proteins such as vWF and incorporated Dil-Ac-LDL. Most convincingly, bone marrow–transplanted genetically tagged cells were covering implanted Dacron grafts.[2] These pioneering studies suggested the presence of circulating hemangioblasts

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From Molecular Cardiology, Department of Internal Medicine IV, University of Frankfurt, Frankfurt, Germany.

Correspondence to Stefanie Dimmeler, PhD, Molecular Cardiology, Dept of Internal Medicine IV, University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. E-mail Dimmeler@em.uni-frankfurt.de

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in the adult. According to the initial discovery, EPCs or CEPCs were defined as cells positive for both hematopoietic stem cell markers such as CD34 and an endothelial marker protein as VEGFR2. Because CD34 is not exclusively expressed on hematopoietic stem cells but, albeit at a lower level, also on mature endothelial cells, further studies used the more immature hematopoietic stem cell marker CD133[3] and demonstrated that purified $CD133^+$ cells can differentiate to endothelial cells in vitro.[4] CD133, also known as prominin or AC133, is a highly conserved antigen with unknown biological activity, which is expressed on hematopoietic stem cells but is absent on mature endothelial cells and monocytic cells (see review).[5] Thus, $CD133^+VEGFR2^+$ cells more likely reflect immature progenitor cells, whereas $CD34^+VEGFR2^+$ may also represent shedded cells of the vessel wall. At present, it is unclear whether CD133 only represents a surface marker or has a functional activity involved in regulation of neovascularization.

Overall, controversy exists with respect to the identification and the origin of endothelial progenitor cells, which are isolated from peripheral blood mononuclear cells by cultivation in medium favoring endothelial differentiation. In peripheral blood mononuclear cells, several possible sources for endothelial cells may exist: (1) the rare number of hematopoietic stem cells, (2) myeloid cells, which may differentiate to endothelial cells under the cultivation selection pressure, (3) other circulating progenitor cells (eg, "side population" cells), and (4) circulating mature endothelial cells, which are shed off the vessel wall[6] and adhere to the culture dishes. First evidence that there is more than one endothelial progeny within the circulating blood was provided by Hebbel and colleagues, who showed that morphological and functional distinct endothelial cell populations can be grown out of peripheral blood mononuclear cells.[7] They stratified the different circulating endothelial cells according to their growth characteristics and morphological appearance as "spindle-like cells," which have a low proliferative capacity, and outgrowing cells. Because the outgrowing cells showed a high proliferative potential and originated predominantly from the bone marrow donors, they were considered as circulating angioblasts.[7] The authors speculated that the spindle-like cells may likely represent mature endothelial cells, which are shed off the vessel wall. However, this hypothesis is difficult to test and has not yet been proven thus far.

Experimentally, preplating may be a way to reduce the heterogeneity of the cultivated EPCs, because this excludes rapidly adhering cells such as differentiated monocytic or possible mature endothelial cells.[2] However, these protocols do not eliminate myeloid and nonhematopoietic progenitor cells, which may contribute to the ex vivo cultivated cells. There is increasing evidence that myeloid cells can give rise to endothelial cells as well. Specifically, $CD14^+/CD34^-$ myeloid cells can coexpress endothelial markers and form tube-like structures ex vivo.[8] Additionally, ex vivo expansion of purified $CD14^+$ mononuclear cells yielded cells with an endothelial characteristic, which incorporated in newly formed blood vessels in vivo.[9] These data would suggest that myeloid cells can differentiate (or transdifferentiate) to the endothelial lineage. Interestingly, lineage tracking showed that myeloid cells are the hematopoietic stem cell–derived intermediates, which contribute to muscle regeneration,[10] suggesting that myeloid intermediates may be part of the repair capacity after injury. Moreover, a subset of human peripheral blood monocytes acts as pluripotent stem cells.[11]

Of note, a specific problem arises when cells are ex vivo expanded and cultured, because the culture conditions (culture supplements such as FCS and cytokines, plastic) rapidly changes the phenotype of the cells. For example, supplementation of the medium with statins increased the number of endothelial cell colonies isolated from mononuclear cells.[12] Moreover, continuous cultivation was shown to increase endothelial marker protein expression.[13] This may explain why different groups may obtain cells with different surface factor profile and functional activity although similar protocols were used for cultivation.[9,14–16] Moreover, the interaction of cells within a heterogeneous mixture of cells such as the mononuclear cells from the blood may impact the yield and the functional activity of the cultivated cells.[17]

Generally, several studies suggested that other cell populations beside hematopoietic stem cells also can give rise to endothelial cells (Figure 1). Thus, non-bone marrow–derived cells have been shown to replace the endothelial cells in grafts.[18] In addition, adult bone marrow–derived stem/progenitor cells such as the side population cells and multipotent adult progenitor cells, which are distinct from hematopoietic stem cells, have also been shown to differentiate to the endothelial lineage.[19,20] Recently, tissue-resident stem cells have been isolated from the heart, which are capable to differentiate to the endothelial lineage.[21] These data support the notion that it will be difficult to define the "true" endothelial progenitor cells. Overall, the field is reminiscent to immunology, where T-cells initially were defined as one cell population. However, the functional characterization (eg, cytokine release and response to stimuli) helped to identify novel T-cell subpopulations with distinct functions and capacities. Hopefully, better profiling of distinct cell populations and fate mapping studies will help to identify markers, which distinguish the circulating endothelial precursor within the blood and bone marrow/non-bone marrow–derived endothelial cells.

Role of EPCs in Neovascularization

Improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia.[22] The finding that bone marrow–derived cells can home to sites of ischemia and express endothelial marker proteins has challenged the use of isolated hematopoietic stem cells or EPCs for therapeutic vasculogenesis. Infusion of various distinct cell types either isolated from the bone marrow or by ex vivo cultivation was shown to augment capillary density and neovascularization of ischemic tissue (Table 1 and Figure 2). In animal models of myocardial infarction, the injection of ex vivo expanded EPCs or stem and progenitor cells significantly improved blood flow and cardiac function and reduced left ventricular scarring.[23,24] Similarly, infusion of ex vivo expanded EPCs deriving from peripheral blood mononuclear cells in nude mice or rats improved the neovascularization in hind limb

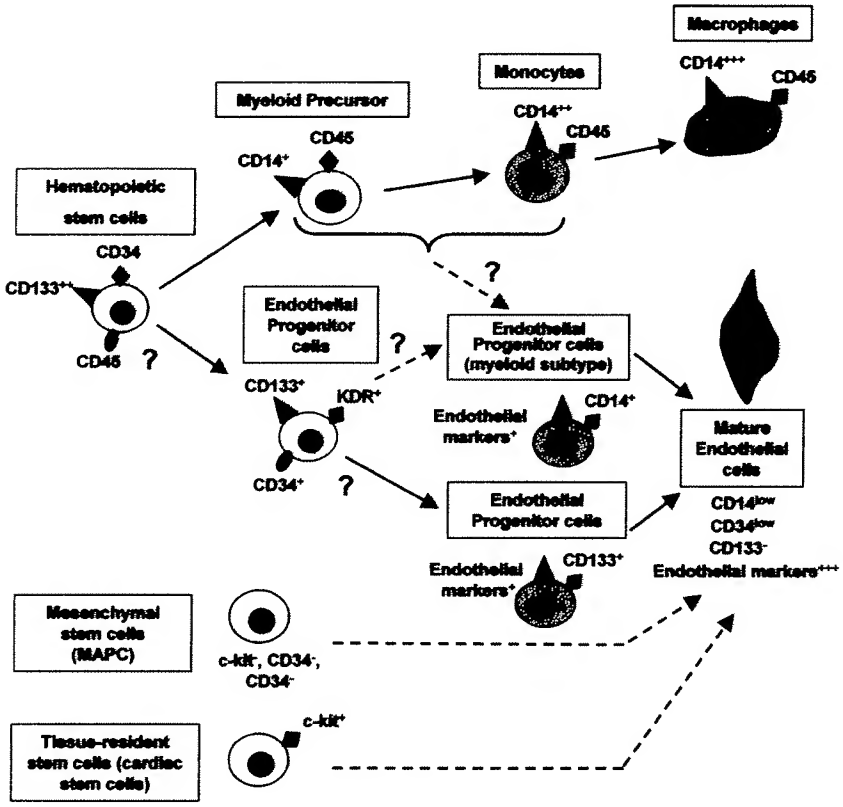

Figure 1. Origin and differentiation of endothelial progenitor cells. Scheme depicts the potential origin and differentiation of endothelial progenitor cells from hematopoietic stem cells and nonhematopoietic cells.

ischemia models.[9,15,23,25] Correspondingly, initial pilot trials indicate that bone marrow–derived or circulating blood–derived progenitor cells are useful for therapeutically improving blood supply of ischemic tissue.[26,27] Autologous implantation of bone marrow mononuclear cells in patients with ischemic limbs significantly augmented ankle-brachial index and reduced rest pain.[26] In addition, transplantation of ex vivo expanded endothelial progenitor cells significantly improved coronary flow reserve and left ventricular function in patients with acute myocardial infarction.[27]

Besides models of peripheral ischemia (hind limb ischemia), the angiogenic potential of EPCs was also investigated in animal models of tumor angiogenesis. Thereby, the inhibition of VEGF-responsive bone marrow–derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth.[28] The use of various different models, cell numbers, and species limits the comparability of the efficiency of distinct cell populations. However, the overall functional improvement appear similar, when isolated human $CD34^+$, $CD133^+$, EPC, MAPC, or murine $Sca\text{-}1^+$ cells were used.[4,9,15,20,23,25,29–32] Likewise, early spindle-like cells and late outgrowing EPCs showed comparable in vivo vasculogenic capacity.[33] These results suggest that the functional activity of the cells to augment neovascularization is rather independent of the type of (endothelial) progenitor cell used. However, the $CD34^-$ fractions of freshly isolated bone marrow– or blood-derived mononuclear cells showed a reduced incorporation and functional activity.[24,29] These data indicate that the number of cells capable to augment neovascularization is low in this crude fraction of freshly isolated uncultivated $CD34^-$ cells. Remarkably, terminally differentiated mature endothelial cells (HMVECs, GEAECs, and SVECs) did not improve neovascularization[15,24,33] suggesting that a not-yet-defined functional characteristic (eg, chemokine or integrin receptors mediating homing) is essential for EPC-mediated augmentation of blood flow after ischemia.

The functional capacity of EPCs to augment blood flow further does not appear to be solely attributable to a monocytic phenotype. Ex vivo cultivated EPCs from $CD14^+$ mononuclear cells or $CD14^-$ mononuclear cell starting population improved neovascularization to a similar extent, whereas the same number of freshly isolated mononuclear cells taken from the starting culture did not.[9] Interestingly, these experimental data are supported by first clinical trials showing that freshly isolated mononuclear cells are not well suited to improve neovascularization in patients with peripheral vascular diseases.[26] However, monocytic cells may play a crucial role in collateral growth (arteriogenesis). Thus, the attraction of monocytic cells by monocyte chemoattractant protein-1 (MCP-1) enhanced arteriogenesis.[34] Moreover, depletion of the monocytes reduced PlGF-induced arteriogenesis.[35] A therapeutic benefit of monocyte infusion on arteriogenesis was demonstrated under conditions of monocyte deficiency induced by chemical depletion.[36] These data suggest that monocytic cells are necessary for arteriogenesis and possibly neovascularization. For therapeutic application, the local enhancement of monocyte recruitment might be better suited than systemic infusion of monocytic cells, which only leads to a relatively minor increase in the number of circulating monocytes.

TABLE 1. Neovascularization Induced by Injection of Progenitor Cells: Experimental and Clinical Studies

Cells	Surface Markers	Improvement Models	Incorporation Rate
Experimental studies			
Freshly isolated cells			
$CD34^+$ cells	$CD34^+$/flk-1^+, $CD45^+$ [1]	Incorporation[1]	13.4 ±5.7% (mouse) or 9.7 ±4.5% (rabbit) Dil-Ac-LDL-EPC in $CD31^+$ capillaries[1]
	Tie-2^+, Dil-Ac-LDL^+ [29]	Hind limb ischemia[29]	Frequently detected (not quantified)[29]
	$CD117^{bright}$/$GATA^-$2/VEGFR2/Tie-2/AC133 [24]	Myocardial infarction[24]	20–25% of total myocardial capillary vasculature[24]
Sca-1^+ BM-MNCs	Sca-1^+ [30]	Hind limb ischemia[30]	Detected (not quantified)
PBMCs	T and B lymphocytes and monocytes-depleted MNCs[30]	Hind limb ischemia[30]	
Ex vivo expanded cells			
Ex vivo expanded EPC	Dil-Ac-LDL^+/lectin$^+$ VEGFR2^+, VE-cadherin$^+$, $CD31^+$, $CD14^+$, $CD34^+$ [15,23]	Hind limb ischemia[15,31] Myocardial infarction[23]	2.1 ±0.4 EPCs into vessels in a ×10 field[15] 241 ±25 cells/mm^2 (day 3) 355 ±30 cells/mm^2 (day 7)[31]
	Dil-Ac-LDL^+, NO^+, VEGFR2^+, VE-cadherin$^+$, $CD31^+$, vWF^+, $CD45^-$ [25]	Hind limb ischemia[25]	Frequently detected (not quantified)[25]
	$CD31^+$, vWF^+, Dil-Ac-LDL^+, VEGFR2^+, Tie-2^+ [53]	Vascular graft survival, Neovessel remodeling[53]	80% of graft lumen at day 15[53]
	Dil-Ac-LDL^+/lectin$^+$ VEGFR2^+, $CD105^+$, vWF^+, $CD45^+$ [9]	Hind limb ischemia[9]	19.8 ±8% $CD146^+$/HLA-DR^+ cell containing vessels[9]
	Early EPC: Dil-Ac-LDL^+/lectin$^+$ VEGFR2^+, $CD31^+$, Tie-2^+, VE-cadherin$^-$, eNOS$^-$, $CD14^+$ [16] Outgrowing ECs: VEGFR2^+, $CD31^+$, Tie-2^+, VE-cadherin$^+$, eNOS$^+$, $CD14^-$ [16]	Matrigel capillaries[16] Outgrowing ECs: exhibited a greater capacity for capillary morphogenesis in in vitro and in vivo matrigel models	ND
	Early EPC: weak VEGFR1, eNOS, vWF, VE-cadherin, VEGFR2, spindle shape[33] Late EPC: strong VE-cadherin, VEGFR1, VEGFR2, eNOS, vWF, cobblestone morphology[33]	In vitro: late EPC showed better incorporation and tube formation. Early EPC: higher release of growth factors. In vivo: comparable vasculogenic potential of early and late EPC (limb perfusion, capillary density)	Detected (not quantified)[33]
MAPC-derived ECs	Co-purified MAPC: $CD34^-$, VE-cadherin$^-$, $AC133^+$, flk-1^+ [20] Angioblast: $CD34^+$, VE-cadherin$^+$, $AC133^-$, flk-1^+ [20]	tumor growth/angiogenesis[20]	MAPC-derived ECs[20] 35% tumor angiogenesis, 30–45% wound healing angiogenesis, undifferentiated MAPCs: 12%
Clinical studies			
BMC and monocytes (TACT-trial)	$CD34^+$/Dil-Ac-LDL^+/lectin$^+$	Intramuscular injection in patients with peripheral ischemic disease; improved blood flow[26]	ND
CPC and BMC (TOPCARE-AMI)	CPC: Dil-Ac-LDL^+/lectin$^+$, VEGFR2^+, $CD31^+$, vWF^+, $CD105^+$; BMC: $CD34^+$/$CD45^+$, $CD34^+$/$CD133^+$, $CD34^+$/VEGFR2^+	Intracoronary infusion in patients with AMI; increase in coronary flow reserve[27]	ND

Mechanisms by Which EPC Improve Neovascularization

Although the role of EPCs in neovascularization has been convincingly shown by several groups, the question remains: how do EPCs improve neovascularization?

Bone marrow transplantation of genetically modified cells (rosa-26, GFP, lacZ) was used to assess the incorporation of bone marrow-derived EPC into tissues. The basal incorporation rate of progenitor cells without tissue injury is extremely low.[37] In ischemic tissue, the incorporation rate of genetically labeled bone marrow–derived cells, which coexpress endothelial marker proteins, differs from 0% to 90% incorporation.[19,28,37–41] Likewise, the extent of incorporation of bone marrow–derived cells in cerebral vessels after stroke varies in the literature.[42–44] Whereas two studies reported positive vessels with an average of 34% endothelial marker expressing bone marrow–derived cells,[42,43] other groups could not detect endothelial marker expressing cells.[44] High amounts (>50%) were predominantly detected in models of tumor angiogenesis.[28,40] Some studies only detected bone marrow–derived cells adjacent to vessels, which do not express endothelial marker proteins.[41,45] A reasonable explanation might be that the model of ischemia (eg, intensity of injury or ischemia)[46] significantly influences the incorporation rate. A minor ischemia might not as profoundly induce a mobilization of bone marrow–derived endothelial progenitor cells and, thus, may lead to a lower percentage of incorporation of bone marrow–derived progenitor cells. The efficiency of engraftment may additionally differ between distinct progenitor subpopulations (pure hematopoietic stem cells versus complete bone marrow cells). Indeed, therapeutic application of cells by intravenous infusion of ex vivo purified bone marrow mononuclear cells or expanded endothelial progenitor cells led to a higher incorporation rate (≈7% to 20% incorporation rate) as compared with the endogenously mobilized bone marrow–engrafted cells (≈2%).[9,47]

However, the number of incorporated cells with an endothelial phenotype into ischemic tissues is generally quite low. How can such a small number of cells increase neovascularization? A possible explanation might be that the efficiency

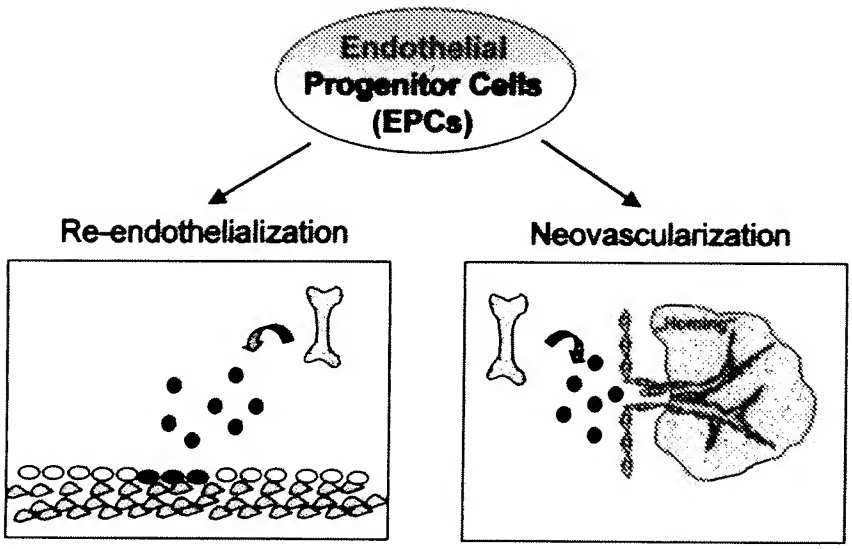

Figure 2. Role of EPCs in vascular biology. Injection of EPCs significantly improve reendothelialization and neovascularization after injury.

of neovascularization may not solely be attributable to the incorporation of EPCs in newly formed vessels, but may also be influenced by the release of proangiogenic factors in a paracrine manner. Indeed, the deletion of Tie-2–positive bone marrow–derived cells through activation of a suicide gene blocked tumor angiogenesis, although these cells are not integrated into the tumor vessels but are detected adjacent to the vessel.[41] Thus, EPCs may act similar to monocytes/macrophages, which can increase arteriogenesis by providing cytokines and growth factors. Indeed, EPCs cultivated from different sources showed a marked expression of growth factors such as VEGF, HGF, and IGF-1 (C.U., unpublished data, 2004). Moreover, adherent monocytic cells, which were cultivated under similar conditions, but do not express endothelial marker proteins, also release VEGF, HGF, and G-CSF.[14] The release of growth factors in turn may influence the classical process of angiogenesis, namely the proliferation and migration as well as survival of mature endothelial cells.[48] However, EPCs additionally incorporated into the newly formed vessel structures and showed endothelial marker protein expression in vivo. In contrast, infusion of macrophages, which are known to release growth factors,[49,50] but were not incorporated into vessel-like structures, induced only a slight increase in neovascularization after ischemia, indicating—but not proving—that the capacity of EPCs to physically contribute to vessel-like structures may contribute to their potent capacity to improve neovascularization.[9] Further studies will have to be designed to elucidate the contribution of physical incorporation, paracrine effects and possible effects on vessel remodeling and facilitating vessel branching to EPC-mediated improvement of neovascularization.

EPCs and Endothelial Regeneration

In the past, the regeneration of injured endothelium has been attributed to the migration and proliferation of neighboring endothelial cells. More recent studies, however, indicate that additional repair mechanisms may exist to replace denuded or injured arteries. Thus, implanted Dacron grafts were shown to be rapidly covered by bone marrow–derived cells deriving from $CD34^+$ hematopoietic stem cells in a dog model.[2] In humans, the surface of ventricular assist devices was covered by even more immature CD133-positive hematopoietic stem cells, which concomitantly express the VEGF-receptor 2.[3] Additionally, Walter and coworkers demonstrated that circulating endothelial precursor cells can home to denuded parts of the artery after balloon injury.[51] Bone marrow transplantation experiments revealed that bone marrow–derived cells can contribute to reendothelialization of grafts and denuded arteries.[51–53] However, in a model of transplant arteriosclerosis, bone marrow–derived cells appear to contribute only to a minor extent to endothelial regeneration by circulating cells.[18] These data again indicate that there might be at least two distinct populations of circulating cells that principally are capable to contribute to reendothelialization, namely mobilized cells from bone marrow and non-bone marrow–derived cells. The latter ones may arise from circulating progenitor cells released by non-bone marrow sources (eg, tissue resident stem cells) or represent vessel wall–derived endothelial cells.[18,51–53]

A rapid regeneration of the endothelial monolayer may prevent restenosis development by endothelial synthesis of antiproliferative mediators such as nitric oxide. Indeed, enhanced incorporation of β-galactosidase–positive, bone marrow–derived cells was associated with an accelerated reendothelialization and reduction of restenosis.[51,52] Similar results were reported by Griese et al, who demonstrated that infused peripheral blood monocyte–derived EPC home to bioprosthetic grafts and to balloon-injured carotid arteries, the latter being associated with a significant reduction in neointima deposition.[54] Likewise, infusion of bone marrow–derived $CD34^-/CD14^+$ mononuclear cells, which are not representing the population of the "classical hemangioblast," contributed to endothelial regeneration.[13] The regenerated endothelium was functionally active as shown by the release of NO,[13] which is supposed to be one of the major vasculoprotective mechanisms. Consistently, neointima development was significantly reduced after cell infusion.[13] Whereas the

regeneration of the endothelium by EPCs protects lesion formation, bone marrow–derived stem/progenitor cells may also contribute to plaque angiogenesis, thereby potentially facilitating plaque instability.[55] However, in a recent study, no influence of bone marrow cell infusion on plaque composition was detected in nonischemic mice.[56] An increase in plaque size was only detected in the presence of ischemia, suggesting that ischemia-induced release of growth factors predominantly accounts for this effect.[56]

Overall, these studies implicate that regardless of the origin of circulating endothelial progenitor cells, this pool of circulating endothelial cells may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing denuded parts of the artery (Figure 2). One can speculate that these cells may also regenerate the low grade endothelial damage by ongoing induction of endothelial cell apoptosis induced by risk factors for coronary artery disease (see review).[57] The maintenance of the endothelial monolayer may prevent thrombotic complications and atherosclerotic lesion development. Although this concept has not yet been proven, several hints from recently presented data are supportive. Thus, transplantation of $ApoE^{-/-}$ mice with wild-type bone marrow reduced atherosclerotic lesion formation.[58] Moreover, various risk factors for coronary artery disease, such as diabetes, hypercholesterolemia, hypertension, and smoking, affect the number and functional activity of EPCs in healthy volunteers[59] and in patients with coronary artery disease.[60] Likewise, diabetic mice and patients were characterized by reduced functional activity of EPCs.[61–63] In addition, factors that reduce cardiovascular risk such as statins[38,51,52,64] or exercise[65] elevate EPC levels, which contribute to enhanced endothelial repair. The balance of atheroprotective and proatherosclerotic factors, thus, may influence EPC levels and subsequently reendothelialization capacity.

Mobilization of EPCs

Because EPCs contribute to reendothelialization and neovascularization, increasing the number of these cells may be an attractive therapeutic tool. The mobilization of stem cells in the bone marrow is determined by the local microenvironment, the so-called "stem cell niche," which consists of fibroblasts, osteoblasts, and endothelial cells (see review).[66] Basically, mobilizing cytokines hamper the interactions between stem cells and stromal cells, which finally allow stem cells to leave the bone marrow via transendothelial migration. Thereby, activation of proteinases such as elastase, cathepsin G, and matrix metalloproteinases (MMPs) cleave adhesive bonds on stromal cells, which interact with integrins on hematopoietic stem cells. MMP-9 was additionally shown to cleave the membrane-bound Kit ligand (mKitL) and induces the release of soluble Kit ligand (KitL; also known as stem cell factor, SCF).[67] Physiologically, ischemia is believed to be the predominant signal to induce mobilization of EPCs from the bone marrow. Ischemia thereby is believed to upregulate VEGF or SDF-1,[68,69] which in turn are released to the circulation and induce mobilization of progenitor cells from the bone marrow via a MMP-9–dependent mechanism.[30,46,67,70] Furthermore, clinical studies using gene therapy with plasmids encoding for VEGF demonstrated an augmentation of EPC levels in humans.[71] Additional factors inducing mobilization of progenitor cells from the bone marrow have been initially discovered in hematology to harvest hematopoietic stem cells from the peripheral blood for bone marrow transplantation. For instance, granulocyte-colony stimulating factor (G-CSF), a cytokine, which is typically used for mobilization of $CD34^+$ cells in patients, also increased the levels of circulating endothelial progenitor cells. A related cytokine, the granulocyte monocyte-colony stimulating factor (GM-CSF), augments EPC levels.[30] Moreover, erythropoietin (EPO), which stimulates proliferation and maturation of erythroid precursors, also increased peripheral blood endothelial progenitor cells in mice[72] and in men.[73] The correlation between EPO serum levels and the number of $CD34^+$ or $CD133^+$ hematopoietic stem cells in the bone marrow in patients with ischemic coronary artery disease further supports an important role of endogenous EPO levels as a physiologic determinant of EPC mobilization.[72] At present, it is not clear which of the mobilizing factors most potently elevates the levels of EPCs. SDF-1 and VEGF165 showed similar effects and rapidly mobilize hematopoietic stem cells and circulating endothelial precursor cells in animal models, whereas angiopoietin-1 induced a delayed and less pronounced mobilization of endothelial and hematopoietic progenitors.[74,75] Whereas a similar increase in white blood cell counts was achieved by G-CSF application, endothelial colonies (CFU-EC) were significantly lower in G-CSF– compared with VEGF- or SDF-1–treated mice. Of note, these data should be interpreted with caution, because the responsiveness toward cytokines may vary between different mice strains and side-by-side comparisons in humans are lacking. Moreover, the extent of increasing neutrophil and lymphocyte levels, which may provoke proinflammatory responses, has to be considered for a potential therapeutic application.

First evidence for potential pharmacological modulation of systemic EPC levels by atheroprotective drugs came from studies using HMG-CoA reductase inhibitors (statins). Statins were shown to increase the number and the functional activity of EPCs in vitro,[38,76] in mice,[38,76] and in patients with stable coronary artery disease.[64] The increase in EPC numbers was associated with increased bone marrow–derived cells after balloon injury and accelerated endothelial regeneration.[51,52] Although statins were shown to increase the number of stem cells within the bone marrow, the mechanism for enhancing EPC numbers and function may additionally include an increase in proliferation, mobilization, and prevention of EPC senescence and apoptosis.[12,38,76] Interestingly, recent studies additionally demonstrated that estrogen increased the levels of circulating EPCs.[77,78] Moreover, exercise augmented EPC levels in mice and in men.[65] The molecular signaling pathways have not been identified thus far. However, several studies indicate that the activation of the PI3K/Akt pathway, which has first been shown to be activated in mature endothelial cells by statins,[79] may also play an important role in statin-induced increase in EPC levels.[12,76] Likewise, VEGF, EPO, estrogen, and exercise are well known to augment the PI3K/Akt-pathway. Thus, these factors may

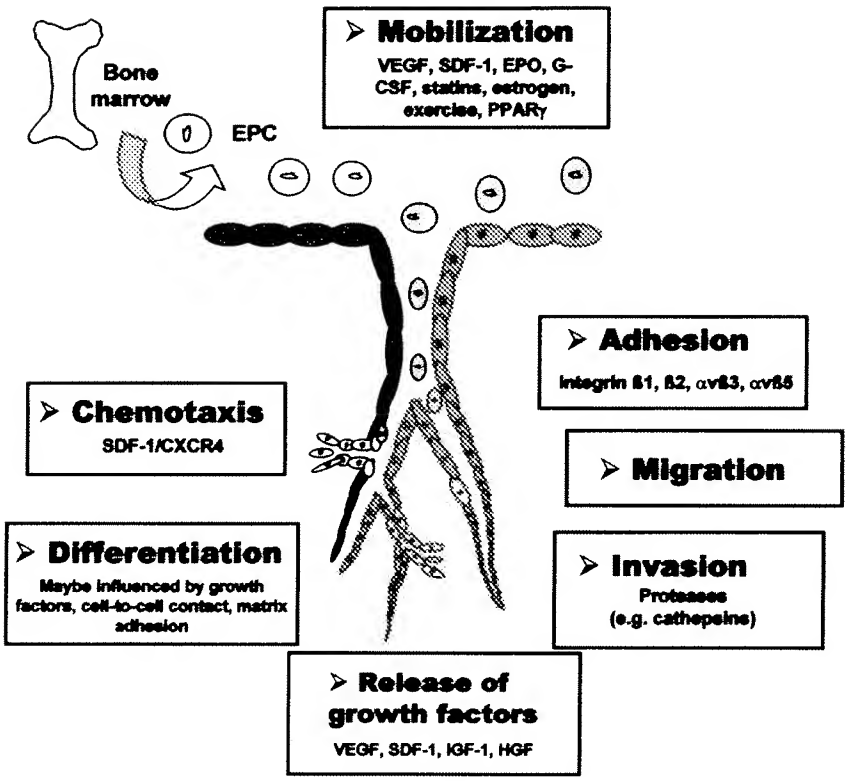

Figure 3. Mechanism of EPC homing and differentiation. Recruitment and incorporation of EPCs into ischemic tissue requires a coordinated multistep process including mobilization, chemoattraction, adhesion, transmigration, migration, tissue invasion, and in situ differentiation. Factors that are proposed to regulate the distinct steps are indicated.

share some common signaling pathways. Given that recent data showed that eNOS is essential for mobilization of bone marrow–derived stem and progenitor cells,[47] one may speculate that these stimuli may increase progenitor cell mobilization by PI3K/Akt-dependent activation of the NO-synthase within the bone marrow stromal cells. Indeed, exercise and VEGF-stimulated EPC mobilization was blunted in $eNOS^{-/-}$ mice.[47,65]

Mechanism of Homing and Differentiation

Although the improvement of adult neovascularization is currently under intensive investigations, the mechanism of homing and differentiation of endothelial progenitor cells is poorly understood. In a previous study assessing in vivo homing of embryonic endothelial progenitor cells derived from cord blood, the circulating cells arrested within tumor microvessels, extravasated into the interstitium, and incorporated into neovessels, suggesting that adhesion and transmigration are involved in the recruitment of endothelial progenitor cells to sites of tumor angiogenesis.[80] Thus, it is conceivable that ex vivo expanded adult EPCs and hematopoietic stem/progenitor cells may engage similar pathways for recruitment to sites of ischemia and incorporation in newly forming vessels. Recruitment and incorporation of EPCs requires a coordinated sequence of multistep adhesive and signaling events including chemoattraction, adhesion, and transmigration, and finally the differentiation to endothelial cells (Figure 3).

Adhesion and Transendothelial Migration

The initial step of homing of progenitor cells to ischemic tissue involves adhesion of progenitor cells to endothelial cells activated by cytokines and ischemia and the transmigration of the progenitor cells through the endothelial cell monolayer.[80] Integrins are known to mediate the adhesion of various cells including hematopoietic stem cells and leukocytes to extracellular matrix proteins and to endothelial cells.[81–83] Integrins capable of mediating cell-cell interactions are the β_2-integrins and the $\alpha_4\beta_1$-integrin. β_1-Integrins are expressed by various cell types including endothelial cells and hematopoietic cells, whereas β_2-integrins are found preferentially on hematopoietic cells.[84] Because adhesion to endothelial cells and transmigration events are involved in the in vivo homing of stem cells to tissues with active angiogenesis,[80] integrins such as the β_2-integrins and the $\alpha_4\beta_1$-integrin may be involved in the homing of progenitor cells to ischemic tissues. Consistent with the high expression of β_2-integrins on hematopoietic stem/progenitor cells, β_2-integrins mediate adhesion and transmigration of hematopoietic stem/progenitor cells.[85,86] β_2-Integrins (CD18/CD11) are expressed on peripheral blood-derived EPCs and are required for EPC-adhesion to endothelial cells and transendothelial migration in vitro (S.D., personal communication, 2004). Moreover, hematopoietic stem cells ($Sca\text{-}1^{+}/lin^{-}$) lacking β_2-integrins showed reduced homing and a lower capacity to improve neovascularization after ischemia (S.D., personal communication, 2004). Interestingly, the homing of inflammatory cells during pneumonia or myocardial ischemia in β_2-integrin–deficient mice is mediated by the $\alpha_4\beta_1$-integrin[87,88] suggesting that deficiency of β_2-integrins can in part be compensated by the $\alpha_4\beta_1$-integrin. Moreover, conditional deletion of the α_4-integrin selectively inhibited the homing of hematopoietic stem/progenitor cells to the bone marrow but not to the

spleen,[89] suggesting that the homing of progenitor cells to different tissues is dependent on distinct adhesion molecules. Furthermore, in vitro studies showed that MCP-1 stimulated adhesion of bone marrow–derived $CD34^-/CD14^+$ monocytes to the endothelium was blocked by anti–β_1-integrin antibodies.[13] Interestingly, in this study, adhesion of $CD34^-/CD14^+$ monocytes isolated from the peripheral blood to endothelial cells was less affected by MCP-1 and was not blocked by anti–β_1-integrin antibodies.[13] Finally, the initial cell arrest of embryonic progenitor cell homing during tumor angiogenesis was suggested to be mediated by E- and P-selectin and P-selectin glycoprotein ligand-1.[80] Yet, it is important to underscore that this study was performed with embryonic endothelial progenitor cells. It is conceivable that different cell types may use distinct mechanisms for homing to sites of angiogenesis.

Cell-cell contacts and transmigration events might be less important for the reendothelialization of denuded arteries (in contrast to homing of progenitor cells to ischemic tissues). With respect to endothelial progenitor cells, studies investigated the contribution of integrins to reendothelialization, which is mainly driven by adhesion to extracellular matrix proteins. Adhesion of EPCs to denuded vessels appears to be mediated by vitronectin-receptors ($\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins). Thus, inhibition of $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins with cyclic RGD peptides blocked reendothelialization of denuded arteries in vivo, suggesting that $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins are involved in the reendothelialization of injured carotid arteries.[51] However, other integrins such as the β_1-integrins may also mediate adhesion of progenitor cells to extracellular matrix proteins during reendothelialization of denuded arteries.[13]

Chemotaxis, Migration, and Invasion

Given the low numbers of circulating progenitor cells, chemoattraction may be of utmost importance to allow for recruitment of reasonable numbers of progenitor cells to the ischemic or injured tissue. Various studies examined the factors influencing hematopoietic stem cell engraftment to the bone marrow. These factors include chemokines such as SDF-1,[90,91] lipid mediators (sphingosine-1-phosphate),[92] as well as factors released by heterologous cells.[93] The factors attracting circulating EPCs to the ischemic tissue may be similar. Indeed, SDF-1 has been proven to stimulate recruitment of progenitor cells to the ischemic tissue.[31] SDF-1 protein levels were increased during the first days after induction of myocardial infarction.[94] Moreover, overexpression of SDF-1 augmented stem cell homing and incorporation into ischemic tissues.[31,94] Interestingly, hematopoietic stem cells were shown to be exquisitely sensitive to SDF-1 and did not react to G-CSF or other chemokines (eg, IL-8 and RANTES).[91] Moreover, VEGF levels are increased during ischemia and capable to act as a chemoattractive factor to EPCs.[68,70,71] Interestingly, the migratory capacity of EPCs or bone marrow cells toward VEGF and SDF-1, respectively, determined the functional improvement of patients after stem cell therapy.[95] Beside genes, which are directly upregulated by hypoxia, the invasion of immune competent cells to the ischemic tissue may further augment the levels of various chemokines within the ischemic tissue, such as MCP-1 or interleukins, which can attract circulating progenitor cells.[13] Whereas several studies shed some light on the mechanisms regulating attraction of EPCs to ischemic tissue, less is known with respect to migration and tissue invasion. One may speculate that proteases such as cathepsins or metalloproteases may mediate the tissue invasion of EPCs.

TABLE 2. Unresolved Questions

How to define an endothelial progenitor cell?
Origin of endothelial progenitor cells?
Definition of subpopulations with different functional capacities?
Signals for EPC homing and differentiation in vivo?
Optimization of ex vivo culture conditions to enhance the benefit of cell therapy?
Influence of the severity of vascular damage on the contribution of EPCs to regeneration?
Mechanisms of action?
Transdifferentiation capacity of different progenitor cells?
Importance of paracrine effects?

Differentiation

Finally, maturation of EPCs to a functional endothelial cell may be important for functional integration in vessels. The genetic cascades that regulate differentiation in the adult system are largely unknown; however, several studies determined the differentiation of the common mesodermal precursor, the hemangioblasts, during embryonic development. Clearly, VEGF and its receptors play a crucial role for stimulating endothelial differentiation in the embryonic development.[96–98] Likewise, VEGF induces differentiation of endothelial cells in ex vivo culture assays using a variety of adult progenitor populations ($CD34^+$,[1] $CD133^+$,[4] peripheral blood mononuclear cells).[15,76] Studies with embryonic stem cells further revealed that the temporal regulation of Homeobox (Hox) genes might play an important role. Thus, the orphan Hox gene termed Hex (also named Prh) is required for differentiation of the hemangioblast into the definitive hematopoietic progenitors and also affected endothelial differentiation.[99] Additionally, the serine/threonine kinase Pim-1 was recently discovered as a VEGF-responsive gene, which contributes to endothelial differentiation out of embryonic stem cells.[100]

Conclusion

Taken together, infusion of different hematopoietic stem cell populations and ex vivo expanded EPCs augmented neovascularization of tissue after ischemia, thereby providing a novel therapeutic option. However, a variety of unresolved questions remain to be answered (Table 2). The crucial question is how to define an endothelial progenitor cell? Overall, there is consensus that endothelial progenitor cells can derive from the bone marrow and that CD133/VEGFR2 cells represent a population with endothelial progenitor capacity. However, increasing evidence suggest that there are additional bone marrow–derived cell populations (eg, myeloid cells) within the blood, which also can give rise to endothelial cells. Moreover, non-bone marrow–derived cells

with endothelial characteristic were isolated from the peripheral blood. This might represent shed mature endothelial cells or other endothelial cells deriving from other progenitor cell populations. Clearly, one functional assay to define endothelial progenitor cells independent of their progeny is the demonstration of clonal expansion activity. Possibly, functional assays will gain additional increasing importance, because recent studies suggest that endothelial progenitor cells have a favorable survival and a better response toward angiogenic growth factors compared with mature endothelial cells.[101] From a therapeutic point of view, these functional activities might be more important than the source of the progenitor cell. Another open question is which mechanism underlies the improvement of neovascularization by infused EPCs? Likely, paracrine effects contribute in addition to the physical incorporation of EPC into newly formed capillaries. The influence of the incorporation of a rather small number of circulating cells on remodeling and vessel maturation has to be further elucidated.

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